Reliability of mRNA Profiling: Verification for Samples with Different Complexities

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ABSTRACT

Normalization of mRNA profiling data remains an open issue, which turns critical when comparing divergent samples or mRNA populations with different complexities. To address this question, we generated samples with different RNA amounts and complexities by subcellular fractionation of cytoplasmic RNA into the mutually exclusive ribosome-free and polysome-bound RNA pools. For each of the 563 mRNAs analyzed, the hybridization signal corresponding to the cytoplasmic sample equals the sum of signals from the ribosome-free plus the polysome-bound targets (cytoplasmic mRNA = ribosome-free mRNA + polysome-bound mRNA). This intuitive equation was fulfilled only after data normalization following "spiking" of the samples with an exogenous RNA. This is the first demonstration that spiking allows one to correct not only for differences in reaction efficiencies but also to reflect the variations in amount and complexity between the initial mRNA populations.

INTRODUCTION

mRNA profiling has become, within a relatively short time period, an invaluable tool for the simultaneous analysis of mRNA expression levels for up to thousands of genes upon physiological, developmental, and pathological processes, as well as in responses to drug treatment (7,11,15,16,20,22,30,33–35). Indeed, new applications and experimental procedures using cDNA arrays are being reported in rapid sequence (9,18,25,31,36,38). Crucial for accurate analysis is the issue of data normalization, which remains to be solved in a satisfactory manner.

In yeast, normalization is usually assessed by hybridization of the target to yeast genomic DNA (8). In higher eukaryotes, this obvious control cannot be applied because, unlike in yeast, only a small part of the genome is expressed (10). Normalization has been performed by using a small subset of housekeeping genes, whose expression levels are presumed to remain unchanged (1,13), or by the mean filter hybridization signal (38). Expression of housekeeping genes is regulated in many circumstances [e.g., the transition from nonproliferating to proliferating cells (3,21,25,39)]. Theoretically, reliable normalization would require one to establish experimentally, for each system, a subset of constantly expressed genes (5). Furthermore, normalization using the average filter hybridization signal is only reliable for closely related samples, in which expression levels for the majority of genes remain unchanged (5). For divergent samples, however, this normalization will also be unreliable because the fraction of genes showing altered transcription rates increases (5) [e.g., during T lymphocyte activation, erythro-

blast differentiation, and early stages of embryonic development, the cells are subject to massive changes in mRNA content and complexity (17,25,29,37)]. In addition, for analysis requiring subcellular fractionation, such as characterization of mRNAs encoding secreted proteins (9) or translationally regulated transcripts (18,25,38), obviously neither normalization using housekeeping genes nor total filter hybridization signals may be reliable.

The translation efficiency of a given mRNA species is characterized by its ribosome loading profile [i.e., its distribution between free mRNPs (messenger ribonucleoprotein particles or ribosome-free mRNAs) and polysome-bound mRNA pools]. In a given cell, each mRNA species is translated with a characteristic efficiency, which may vary more than 100-fold for the different cellular mRNAs. Furthermore, translation efficiency of a given mRNA may vary under different cellular conditions (e.g., stimulation, proliferation, differentiation, or drug treatment) (25). Such a regulation is an essential mechanism to control gene expression because it may affect more than 10% of all expressed mRNA species (12,25).

We have addressed the reliability of array hybridization analyses for the comparison of mRNA populations of different amount and complexities, by comparing for each transcript the signals obtained from the cytoplasmic mRNA population with the signals obtained from the two mutually exclusive ribosome-free (untranslated) and polysome-bound (translated) mRNA populations. Statistical analysis reveals that, for each individual cDNA in the array, the signal intensity from the cytoplasmic mRNA population indeed represents the sum of the signal intensities from the ribosome-free plus the

polysome-bound samples, after normalization of the values with an exogenous RNA. This is the first formal demonstration that “spiking” of the samples is suitable for quantitative comparisons between RNA populations of different complexities.

MATERIALS AND METHODS

Cells, RNA Preparation, and Sucrose Gradients

The murine cytotoxic T cell clone B6.1.SF.1 (B6.1) (14,37) was cultured in DMEM-10 [DMEM supplemented with 10% heat-inactivated FCS, 20 mM HEPES, pH 7.0, 0.05 mM β -mercaptoethanol, 2 mM glutamine, and 1% X63mIL-2 supernatant as a source of recombinant IL-2 (19)]. Cells in exponential growth were harvested and used either to prepare cytoplasmic mRNA using the NP40 method (28) or for subcellular fractionation in linear 15%–40% sucrose gradients (27).

Aliquots of RNA purified from each fraction were separated through denaturing formaldehyde-agarose gels and transferred to nylon membranes (Gene-Screen[®]; NEN[®] Life Science Products, Boston, MA, USA). rRNA distribution was visualized by methylene blue staining of the filters (26). Northern blots were hybridized with random-primed [α -³²P]dCTP-labeled cDNA probes. Signals were quantified by phosphorimaging using ImageQuant[™] software (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

cDNA Synthesis, Labeling, Hybridization, and Data Analysis

Fractions 1–10 (containing free ribonucleoprotein particles) and fractions 11–19 (corresponding to polysome-bound mRNA) from the sucrose gradient were pooled. Poly(A⁺) mRNAs were purified from these pools and from cytoplasmic RNA, using Oligotex-dT[™] beads (Qiagen, Hilden, Germany) as described (28). Quality and extraction yields were analyzed by filter hybridization after electrophoresis. For subsequent reverse transcription, one-fourth of each poly(A⁺) mRNA extracted from the ribosome-free and

polysome-bound mRNA pools was used, as well as a fraction of poly(A⁺) mRNA from the cytoplasmic RNAs, equivalent to the free plus bound material. Five nanograms of heterologous, polyadenylated exogenous RNA, obtained by in vitro transcription of the plasmid pBluescript[®]-HS59 (a kind gift of J. Paz Ares, CNB-CSIC, Madrid, Spain), containing the cDNA HS59 from *Arabidopsis thaliana*, were added to each poly(A⁺) mRNA sample before cDNA synthesis. Reverse transcription was performed using oligo-dT (Life Technologies, Rockville, MD, USA) as described (24). Reaction products were purified on Sephadex[®] G-50 spin columns (Roche Molecular Biochemicals, Indianapolis, IN, USA) and the amount of first-strand cDNA quantified by fluorometry (DyNAquant[™]200; Amersham Pharmacia Biotech). Forty-five nanograms of first-strand cDNA were used to generate [α -³³P]dATP-labeled second-strand cDNA. These radiolabeled targets were used to hybridize four Atlas mouse cDNA arrays (consecutive serial numbers; Clontech Laboratories, Palo Alto, CA, USA), essentially as described (26), using 1 μ g/mL mouse C₀t-1 DNA as competitor. A small filter containing an excess (200 ng) of pBluescript-HS59 plasmid DNA was simultaneously hybridized with each array. Signals were quantified by phosphorimaging using ImageQuant software. Since each cDNA was present in duplicate on the filters, the mean value was calculated. cDNA for which the hybridization values did not verify for the three filters that: $0.7 \times \text{mean}_{x,y} < x, y < 1.3 \times \text{mean}_{x,y}$, were excluded from further analysis (25 cDNAs in all). Linear regression analysis and correlation coefficients were calculated using Microsoft[®] Excel[®] 98.

RESULTS AND DISCUSSION

Subcellular RNA Fractionation and Characterization

RNAs from cytoplasmic extracts were fractionated by sedimentation velocity in 15%–40% sucrose gradients. In these gradients, migration of ribosome-bound transcripts is directly proportional to their ribosome loading,

while free mRNAs (in the form of mRNPs) migrate at the top of the gradient (23). The characteristic profile of rRNA distribution in sucrose gradients can be visualized by methylene blue staining of the northern blot (Figure 1A), where the migration of the 40S small ribosomal subunit (peak of 18S rRNA in fractions 5 and 6) and the migration of the 60S big ribosomal subunit (peak of 28S rRNA in fractions 8 and 9) are visualized. Consequently, it appears that mRNA molecules migrating in fractions 1–9 are not engaged in translation, whereas mRNAs migrating in more dense fractions are loaded with one or more ribosomes and, thus, are being actively translated.

The translational efficiency for each mRNA can be determined by its distribution pattern in sucrose gradients. Indeed, eEF1 α mRNA, coding for the translation elongation factor 1 α , shows the typical profile for an inefficiently translated transcripts because most of this mRNA is found in the ribosome-free fractions (Figure 1, B and C). Conversely, β -actin mRNA exhibits a profile characteristic of an efficiently translated transcripts; it is mostly bound to polysomes (Figure 1, B and C). The ratio ribosome-free:polysome-bound is an indication of the translation efficiency of the transcript. Here, this ratio was 3.42 and 0.08 for eEF1 α and β -actin mRNAs, respectively (Figure 1). Several hybridization experiments using cDNA arrays have demonstrated that the ribosome-free:polysome-bound ratios usually range from 10 to 0.08 (25) (additional information at <http://www.cnb.uam.es/~jasanz/pradet2000a>), indicating that each mRNA species has a specific, independent distribution between these two mutually exclusive pools.

cDNA Array Hybridization and Analysis

Array hybridization experiments using cytoplasmic and fractionated ribosome-free and polysome-bound mRNA populations were then performed. Following sucrose gradient fractionation, RNAs from fractions 1–9 (ribosome-free) and from fractions 10–19 (polysome-bound) were pooled. Fraction 20 is routinely discarded because, in addi-

Research Report

tion to polysomal mRNAs, it also contains mRNAs attached to high-density cell debris migrating at the bottom of the gradient. Poly(A)⁺ mRNAs purified from the three RNA populations (ribosome-free, polysome-bound, and cytoplasmic) were used to generate targets for the hybridization of three identical mouse cDNA arrays (Clontech Laboratories) (Figure 2A). After signal quantification, cDNAs whose duplicate hybridization signals deviated more than 1.3-fold from the mean value in all three filters were discarded. This excluded 25 out of the 588 cDNAs in the arrays. The dynamic range of signal intensity for the remaining 566 cDNAs was similar for the three mRNA populations, spanning more than three orders of magnitude (Figure 3).

So far, reliability of cDNA array analysis has only been addressed by repeated hybridization of the arrays with the same targets or with targets derived from the same mRNA population, thus demonstrating the reproducibility of the system (2,32). To address the question of reliability in the comparison of divergent mRNAs, we took advantage of the following facts: (i) the three RNA populations used here have intrinsically different amounts and complexities; (ii) the ribosome-free and polysome-bound RNA populations are mutually exclusive and represent truly independent mRNA populations because each mRNA species has a distinct, characteristic distribution between the two pools; (iii) for each mRNA analyzed, the signal intensity obtained from hybridization with the cytoplasmic targets should be equal to the sum of the signal intensities obtained after hybridization with the ribosome-free and polysome-bound targets. This relation becomes, after a logarithmic transformation (to facilitate graphical representation of the data):

$$\log_{10}(\text{cytoplasmic mRNA}) = \log_{10}(\text{ribosome-free mRNA} + \text{polysome-bound mRNA})$$

where "mRNA" stands for the abundance of any individual transcript in the indicated pool.

Regression analysis of the data obtained after hybridization of these samples showed a significant correlation coefficient ($r^2 = 0.972$) (Figure 2B) to a

line defined by the following equation:

$$y = 0.986x + 0.728$$

This regression line, although it has a slope close to 1, does not intersect the coordinate origin, demonstrating that the signals obtained with the cytoplasmic mRNA samples were only proportional to the sum of the ribosome-free plus polysome-bound signals. In other words, for each cDNA, the hybridization signals obtained with each of the three mRNA populations cannot be directly compared. Thus, the variations between the hybridization signals would not be representative of the genuine variations in mRNA expression level in the experiment.

Normalization of Hybridization Data

To improve data analysis, normalization was assessed by spiking the initial samples with an heterologous poly(A)⁺-tailed transcript generated in vitro from an *A. thaliana* cDNA. The hybridization signal corresponding to this exogenous RNA allowed us to calculate a specific correction factor for each array (K_C for hybridization with the target corresponding to the cytoplasmic RNAs, K_F the ribosome-free, and K_B the polysome-bound) (Figure 2C). After correction of the hybridization signals, the analysis (Figure 2D) yielded the same correlation coefficient ($r^2 = 0.972$) of the data to a line with the following equation:

Figure 1. Analysis of RNA fractionation in sucrose gradients. Cytoplasmic extracts from B6.1 cells in exponential growth were layered on top of a 15%–40% linear sucrose gradient. After centrifugation, 20 fractions were obtained from the sample, and RNA was purified from each of them. (A) Methylene blue staining of the filter after electrophoretic separation of RNA from each fraction and transfer to a nylon membrane. The migration of 28S, 18S, and 5S rRNAs is indicated. Fraction 1 corresponds to the top, fraction 20 to the bottom. (B) Hybridization signals for eEF1α and β-actin mRNAs show typical distributions for either inefficiently (eEF1α) or efficiently (β-actin) translated mRNAs. (C) Distribution of eEF1α and β-actin mRNAs across the sucrose gradient. Data from (B) were quantified by phosphorimaging and plotted as percentage of the total signal, to allow direct comparison between the profiles of two genes, expressed at different levels.

Figure 2. cDNA array hybridization with ribosome-free, polysome-bound, and cytoplasmic B6.1 RNA. mRNA samples were prepared from the ribosome-free pool (fractions 1–9), polysome-bound (fractions 10–19), or cytoplasmic RNA from B6.1 cells. After poly(A)⁺ purification, 5 ng exogenous RNA obtained by in vitro transcription of the cDNA HS59 from *A. thaliana* were added to each sample for normalization purposes. Each of these mixtures was used to prepare a complex cDNA target, which was then hybridized to a cDNA array (Clontech Laboratories), as described in Materials and Methods. (A) Signals obtained after hybridization of cDNA arrays with the ribosome-free, polysome-bound, and cytoplasmic targets (PhosphorImager® scale 0.07–5000; Amersham Pharmacia Biotech). (B) Scatter plot for each of the 563 genes analyzed, representing the signal with the cytoplasmic target versus the sum of the signals from free plus bound targets, after logarithmic transformation of the data values. (C). Hybridization signals for each target with a plasmid containing the *A. thaliana* HS59 cDNA (several bands represent the different mobility forms of the plasmid). The correction factor for each sample was calculated by dividing the exogenous RNA-specific signal from the polysome-bound sample (the highest signal) by the exogenous RNA-specific signal for each target used. (D) Scatter plot representing the signal from the corrected cytoplasmic target versus the sum of the corrected signals from free plus bound targets after correction.

Research Report

$$y = 0.986x + 0.189$$

The regression line has still a slope close to one, and this time it intersects the coordinates very close to the origin. This means that, for each cDNA analyzed, the hybridization signals are consistent with the intuitive equation that relates the three mRNA populations:

$$\text{cytoplasmic mRNA} = \text{ribosome-free mRNA} + \text{polysome-bound mRNA}$$

Therefore, normalization by spiking of the sample allows a quantitative analysis of mRNA profiling data independently of the amount and complexity of the mRNA in the populations analyzed and allows direct comparison of the data.

Different complexities and changes in RNA abundance may be due to sub-cellular RNA fractionation but also be the result of physiological processes. Indeed, massive transcription bursts take place, for example, following macrophage and lymphocyte activation (4,6) and during early development

(37). Conversely, generalized transcriptional repression takes place during terminal differentiation processes such as terminal erythropoiesis (29). For these examples, it is crucial that the RNA populations compared reflect the initial cellular conditions. Especially since, because of technical requirements, the same amounts of material are often used for some of the reaction steps, which inevitably results in misrepresentation of the initial sample complexity and amount. Our data demonstrate, for the first time, that spiking of the RNA populations allows one to compensate for this distortion.

Obviously, the exogenous RNA should not hybridize with either the sample RNA to be analyzed or the cDNA array. For this purpose, an *A. thaliana* RNA, which does not hybridize with either murine (data presented here) or human (BP-B and JAG-S; data not shown) cDNA arrays, was used. Furthermore, spiking might also be used for comparisons of samples that cannot be simultaneously hybridized and exposed

because of technical limitations. The use of such exogenous RNA for data normalization is readily feasible at low cost, both in terms of time and reagents required. In contrast, all other steps in expression profiling, from RNA extraction to array data analysis, are rather expensive and time consuming.

In conclusion, mRNA profiling emerges as a powerful tool for simultaneous analysis of mRNA expression levels for hundreds to thousands of genes (11). Crucial to data analysis is the issue of accurate normalization, particularly for comparison between divergent samples, which might generate RNA populations with different amounts and complexities. Here, we show that the accurate use of an exogenous RNA for spiking of the samples allows one not only to ensure reproducibility for target synthesis and hybridization but also to restore the initial variations between sample complexities and amounts.

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Figure 3. Dynamic range of cDNA array hybridization signals using different targets. Specific hybridization signals for each filter were sorted by increasing values and plotted against a running index in the x-axis. The dynamic range of the signal intensities after hybridization with cytoplasmic, ribosome-free, or polysome-bound targets spans over four orders of magnitude in all cases.

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